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Nitrogen Metabolism of Lemna minor. II. Enzymes of Nitrate Assimilation and Some Aspects of Their Regulation[1]

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Abstract. In *L. minor* grown in sterile culture, the primary enzymes of nitrate assimilation, nitrate reductase (NR), nitrite reductase (NiR) and glutamate dehydrogenase (GDH) change in response to nitrogen source. NR and NiR levels are low when grown on amino acids (hydrolyzed casein) or ammonia; both enzymes are rapidly induced on addition of nitrate, while addition of nitrite induces NiR only. Ammonia represses the nitrate induced synthesis of both NR and NiR.

NADH dependent GDH activity is low when grown on amino acids and high when grown on nitrate or ammonia, but the activities of NADPH dependent GDH and Alanine dehydrogenase (AlDH) are much less affected by nitrogen source. NADH-GDH and AlDH are induced by ammonia, and it is suggested that these enzymes are involved in primary nitrogen assimilation.

Assimilation of nitrate, the primary nitrogen source for many plants, involves reduction by the enzymes nitrate reductase and nitrite reductase. The resulting ammonia is then incorporated into organic form, and glutamate dehydrogenase is one of the key enzymes in this process, the amino nitrogen subsequently being distributed to other amino acids by aminotransferase activity.

While there are many reports of regulation of enzyme synthesis for microorganisms, examples from higher plant tissue are less common. However, regulation of some of the enzymes of nitrate assimilations does occur. Induction of nitrate reductase has been reported for corn (8), radish (4) and cauliflower (1), while nitrite reductase induction has been demonstrated in radish (4) and corn (7). The control of these enzymes through repression is less well documented. Although ammonia appeared to repress nitrate reductase synthesis in *Chlorella* (11) it was found to promote induction, by nitrate, of this enzyme in radish cotyledons (4). Filner (2) has shown that nitrate reductase is repressed by amino acids in cultured tobacco cells, but this could not be demonstrated for higher plant tissue (see 5). Difficulties arising from bacterial contamination have curtailed extensive work on this aspect. Less is known of the control of glutamate dehydrogenase. In radish no rapid changes in enzyme level were detected, although there was some indication that long term changes occurred in response to different sources of nitrogen (4). Regulation of the enzyme by amino acids has been shown in yeast (9).

Lemna is a higher plant, but can be maintained quite easily in sterile culture. This paper reports investigations on the primary enzymes of nitrogen assimilation in *Lemna*, and their response to use of different nitrogen sources, and some aspects of control of these enzymes are described. Some of the properties of the *Lemna* system have been outlined in an accompanying paper (5).

Materials and Methods

Culture Conditions. Lemna minor was grown under sterile conditions, using 200 ml medium in 1 liter flasks, as described earlier (5). The medium contained minerals and sucrose (1% w/v), and nitrogen was supplied as either nitrate [KNO_3 15 mM, $Ca(NO_3)_2$ 5 mM] or ammonia [$(NH_4)_2SO_4$ 7.5 mM] or hydrolyzed casein (3 g/l, BDH Oxoid, acid hydrolyzed).

In some induction studies, cultures were resuspended in fresh medium. This was achieved by replacing the flask plug with a sterile plug assembly containing a glass tube which reached to the bottom of the flask. Used culture medium was aspirated from the culture flask through the tube, a pad of cheesecloth preventing entry of plants into the tube. In later experiments the medium was removed through a side arm, fitted with a fritted glass disc, sealed to the base of the flask. Fresh sterile medium was then added to the flasks. In other induction studies a small volume of sterile solution containing the inducing substance was added to the existing culture medium. These operations were carried out in a sterile room.

Enzyme Preparation. Cultures were harvested after 28 to 33 days growth, giving at least 5 g of fresh material per flask. The plants were collected

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on cheesecloth, rinsed several times with distilled water, then after shaking off loose water were lightly pressed between pads of paper towelling for 20 sec. The tissue was weighed, and 5 g of material was homogenized for 15 to 20 sec in a Virtis homogenizer with 12.5 ml of cold phosphate buffer (pH 7.5, 0.05 M). In some experiments EDTA (final concn 0.01 M) or Clelands reagent (dithiothreitol, final concn 1 mM) was added to the homogenizing buffer. The homogenate was poured through 4 layers of cheesecloth and centrifuged at 1000*g* for 5 min. Non-ionic detergent (Tergitol NPX, Union Carbide) was added to the supernatant to give a final concentration of 0.1 %, and the solution was allowed to stand for 10 min to aid breakdown of particle membranes. The solution was finally clarified at 29,000*g* for 10 min and the supernatant was assayed for enzyme activity. Nitrate reductase was relatively unstable and was assayed immediately after final centrifugation. All preparations were maintained at 0 to 4°, and glass distilled water was used throughout the work.

Enzyme Assays. Nitrate reductase (E.C. 1.6.6.1) was assayed by measuring NADH dependent production of nitrite (3).

Nitrite Reductase (in Leaves: Reduced Ferredoxin:Nitrite Oxidoreductase). Disappearance of nitrite using dithionite as electron donor was assayed as described previously (6) but substituting methyl viologen for benzyl viologen.

Glutamate and Alanine Dehydrogenases. The rate of oxidation of NAD(P)H, dependent on presence of ammonia and the appropriate α-keto acid, was measured by continuous recording of E_{340} in a Zeiss PMQII spectrophotometer. The complete assay system contained 0.2 ml enzyme; 1.9 ml buffer (see below); 0.2 ml 1 M ammonium sulfate; 0.2 ml 0.2 M α-ketoglutarate or pyruvate (adjusted to pH 7.5). The reaction was started by adding 0.5 ml 0.1 mM NAD(P)H, made up in water brought to pH 7.5 by addition of a trace of phosphate buffer. Assay buffers used were: NADH-GDH 0.2 M tris, pH 8.4; NADPH-GDH 0.1 M phosphate, pH 7.5; Alanine DH (E.C. 1.4.1.1, NADH activity only) 0.1 M phosphate, pH 7.5. Blanks lacking α-keto acid or ammonia were run for each assay.

Other Enzymes. It was not possible to assay aspartate dehydrogenase in crude preparations, since addition of oxaloacetate caused a rapid oxidation of NADH, not dependent on ammonia, presumably due to the presence of malate dehydrogenase. The presence of an active aspartate aminotransferase was inferred from noting a rapid NADH oxidation dependent on the presence of α-ketoglutarate with aspartate. Presumably oxaloacetate released in the transamination was reduced by the malate dehydrogenase system.

Enzyme Units. 1 mU of enzyme catalyzed the transformation of 1 mμ mole of substrate per min.

Nitrate was assayed by the method of Woolley, Hicks, and Hageman (12).

Results

Enzyme Levels. The activity of enzymes of nitrate assimilation in *Lemna* grown on various nitrogen sources is shown in table I. Nitrate and nitrite reductases were present in high concentrations when nitrate was supplied to the plants, but only traces of activity were found in tissues grown on ammonia or amino acids. NADH dependent glutamate dehydrogenase was high with nitrate as nitrogen source, low with amino acids and intermediate with ammonia. NADPH dependent glutamate dehydrogenase and alanine dehydrogenase were relatively unchanged by nitrogen source, but did show a graduation from highest levels with nitrate to lower levels with amino acids. These results strongly suggest that the nitrogen source regulates the level of nitrate and nitrite reductases and NADH dependent glutamate dehydrogenase. It should be noted that the maximum activity of nitrate reductase was much lower than any of the other enzymes which would be involved in nitrate reduction and assimilation.

Table I. *Effect of Nitrogen Sources on Enzyme Levels of* L. minor

Cultures were grown 31 days with amino acids (hydrolyzed casein), nitrate or ammonia as nitrogen source. Enzyme activities are expressed as mU/g fresh material. Results are the average of determinations from 2 replicate cultures.

N Source:	Amino acids	Ammonia	Nitrate
	mU per gram fresh wt.		
Nitrate reductase	0.4	0.4	14.6
Nitrite reductase	8	11	267
Glutamate DH - NADH	29	220	524
Glutamate DH - NADPH	68	95	127
Alanine DH - NADH	30	43	51

Enzyme Induction. Changes in enzyme levels were investigated when *Lemna* cultures were transferred from one nitrogen source to another. Cultures were grown 29 days on amino acid media, since all enzymes showed lowest activity in this medium, and were then transferred to ammonia or nitrate media. Cultures were harvested and assayed after 1, 2, and 3 days further growth on the substituted nitrogen source. Results are shown in Fig. 1, a-d. Nitrate reductase was rapidly induced by nitrate, but not by ammonia, and after 24 hr the enzyme showed higher activity than if the plants had been grown continuously on nitrate (26 mU/g fresh wt compared with 14.6). Nitrite reductase changes (not shown) followed closely the pattern shown by nitrate reductase. Both NADH-glutamate dehydrogenase and alanine dehydrogenase showed only a slight increase with nitrate, but ammonia caused a marked stimulation of both these enzymes. The rate of induction was slower than that of nitrate reductase, showing

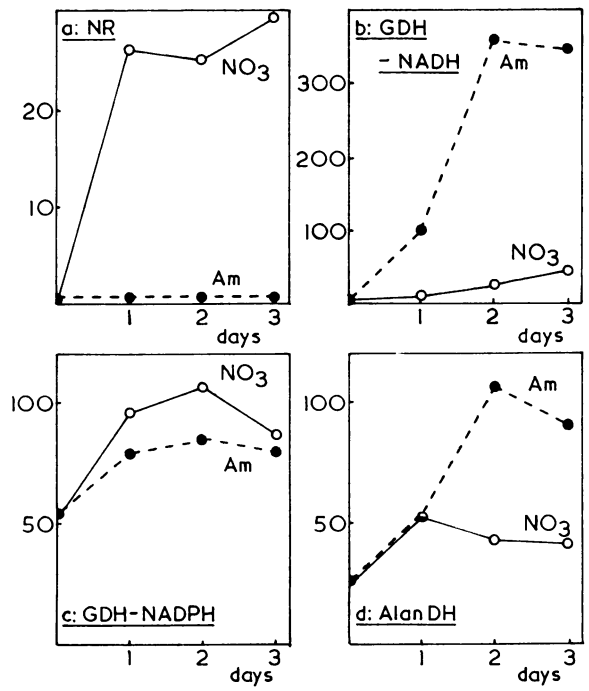

FIG. 1. Effect of nitrogen source on enzyme induction in *L. minor*. Cultures grown 29 days on amino acids then transferred to ammonia (- - - -) or nitrate (———) media. Ordinate axis units are mU enzyme/g fresh weight. a) Nitrate reductase; b) NADH dependent glutamate dehydrogenase; c) NADPH dependent glutamate dehydrogenase; d) Alanine dehydrogenase.

high activity only on the second day. The activity of NADH-glutamate dehydrogenase induced by ammonia was not quite as high as when tissues were grown continuously on nitrate (366 mU/g fresh wt compared with 524), but for alanine dehydrogenase the ammonia induced level exceeded the continuous nitrate level (106 mU/g fresh wt compared with 51).

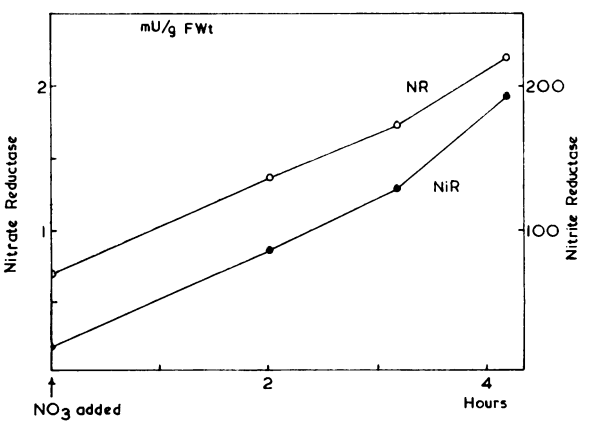

FIG. 2. Time course of induction of nitrate reductase (NR), and nitrite reductase (NiR) after addition of nitrate to *L. minor* grown on amino acid media.

NADPH dependent glutamate dehydrogenase showed a small increase with both nitrate and ammonia.

The time course of nitrate and nitrite reductase induction was investigated at shorter time intervals, as shown in Fig. 2. The induction was rapid and after only 2 hr considerable increases in activity are detectable.

The process appeared to be true induction, with *de novo* synthesis of protein. The presence of cycloheximide (2 mg/l) added with nitrate caused a 93 % inhibition of nitrate reductase induction. This was not caused by interference with nitrate transport, since the level of nitrate in the inhibited tissues was almost twice that found in tissues without cycloheximide in which nitrate reductase was induced normally (92 μg NO_3-N/g fresh wt in the inhibited tissue, compared with 59 in normal tissue).

Induction by Nitrite. It was not possible to determine from a previous experiment (Fig. 2) whether the induction of nitrate and nitrite reductases was simultaneous in response to nitrate, or was sequential. However it was shown that nitrite reductase was substrate inducible and could be synthesized in the absence of nitrate reductase. Results of an experiment comparing nitrate and nitrite as inducers is shown in table II. With nitrate both enzymes were induced, but nitrite caused induction of nitrite reductase only and nitrate reductase remained at a low level.

Table II. *Specificity of Enzyme Induction by Nitrate and Nitrite, in* L. minor

Cultures were grown 28 days with amino acids as nitrogen source, then transferred to nitrate (5 mM) or nitrite (0 5 mM) media. Enzymes were assayed after a 24 hr induction period.

	Nitrate reductase	Nitrite reductase
	mU per gram fresh wt.	
No addition	0.71	39
+ Nitrate	12.7	310
+ Nitrite	1.3	279

Nitrite was quite toxic to the tissues. Under some conditions higher concentrations were tolerated (5 mM) and in these cases nitrite reductase was very active and was apparently able to reduce all incoming nitrite, since free nitrite was not detectable within the tissue. At higher levels of nitrite, nitrite reductase activity was lower and nitrite accumulated in the tissues, and the plants died within a few days. The plants were much more susceptible to nitrite toxicity when amino acids were also present in the medium.

Enzyme Repression. Evidence of repression of nitrite and nitrate reductases was found when nitrate was added as inducer in the presence of varying concentrations of ammonia. Results of 1 such experiment are shown in table III. While 1 mM ammonia had a little effect on the reductases, 2 and

Table III. *Repression of Nitrate and Nitrite Reductases by Ammonia*

Cultures of *L. minor* were grown 29 days with amino acids as nitrogen source, then transferred to media containing ammonia at different concentrations. After 24 hr in presence of ammonia, nitrate (final concentration 2 mM) was added and enzymes assayed after a further 24 hr induction period. For treatment 1, nitrate was added directly to an amino acid grown culture (for 24 hr). Results are averaged from 2 replicate treatments.

	Nitrate reductase	Nitrite reductase	Glutamate dehydrogenase (NADH)
	mU per gram fresh wt.		
1. NO_3^- only	12.0	475	26.5
2. 1mM NH_4^+, + NO_3^-	11.4	455	37.1
3. 2mM NH_4^+, + NO_3^-	8.7	291	68.9
4. 4mM NH_4^+, + NO_3^-	7.1	258	143.0

4 mM caused 28 % and 41 % decreases respectively for nitrate reductase, and 39 % and 46 % decreases for nitrite reductase. At the same time glutamate dehydrogenase in the tissue increased with ammonia concentration, suggesting that protein synthesis could occur, and that the repressive effect of ammonia was not due to some general inhibitory effect on metabolism in general.

Discussion

The complexity of the multicellular organism complicates the study (and presumably the actual operation) of control mechanisms, compared with the situation in microorganisms. For higher plants the raw materials utilized in growth are rigidly defined in some areas of metabolism, and there is little biological advantage in a flexible system of regulated enzymes, since alternative sources of nutrition are unlikely to be available. But nitrogen metabolism is an example of an area of metabolism where alternative metabolites may be available to the plant, and thus regulation mechanisms are of value.

In *Lemna* the enzymes of nitrate and ammonia assimilation are inducible, and the activities change considerably in response to nitrogen source. Nitrate and nitrite reductases are induced by nitrate, and ammonia induces glutamate and alanine dehydrogenases, enzymes which it is reasonable to assume are important in the process of assimilation of nitrogen into organic form. Induction of nitrate reductase is well known, and has already been discussed in the introduction. Recent work by Stewart (10) describes induction of nitrate reductase in *Lemna*, but the rate of induction was slow, slight increases of enzyme activity being detected after 5 to 10 hr in the presence of nitrate, and maximum activity was not obtained until 60 to 70 hr. In the present work substantial amounts of enzyme were detected after 2 hr in nitrate, and induction was more or less complete after 24 hr. The method of growth (continuous culture) used by Stewart may explain the difference in induction rates.

When cultures grown on amino acids were transferred to nitrate medium, the level of nitrate reductase induced was higher (Fig. 1) than activity in cultures of similar age that had been grown continuously on nitrate, supplied initially at the same concentration (table I). This is explained in part by the fact that the enzyme level is dependent on substrate concentration, and plants grown in nitrate would have utilized some of the available nitrate, giving concentrations lower than in the newly supplied nitrate medium. This was confirmed when it was found that transfer of nitrate grown plants from old to fresh medium caused an increase in activity of nitrate reductase. It is also likely that some overshoot of newly induced enzyme occurs before the tissue reaches a steady state on the new nitrogen medium.

The levels of nitrate reductase assayed in the tissue were at least an order of magnitude lower than activities of enzymes involved later in the pathway of nitrate assimilation. As this is a very labile enzyme, it is possible that only a fraction of the enzyme was extracted and assayed; however, the activities and ratios to other enzymes have been reproducible in many different experiments. An enzyme of low activity at the beginning of a metabolic pathway allows that enzyme to control the flow of metabolite into that pathway, and it may be that nitrate reductase has this role.

Nitrite reductase was also inducible in the tissue, and behavior was very similar to that of nitrate reductase, except that nitrite reductase was induced by nitrite as well as by nitrate. It was not possible to determine whether induction of nitrite reductase by nitrate was simultaneous with nitrate reductase or whether a sequential induction occurred.

For both nitrate and nitrite reductases repression-like effects were produced by ammonia. Control of nitrate reductase by ammonia has been shown in *Chlorella* (11). In some other experiments with radish (4) and corn (8) no such repressive effect was demonstrated, although these investigations did not include pretreatments with a series of ammonia concentrations prior to induction.

NADH dependent glutamate dehydrogenase is induced by its substrate ammonia, and as far as is known, this is the first indication of its inducibility in plant tissue. The response is proportional to ammonia concentration (table III). Alanine dehydrogenase activity is also induced by ammonia, and this behavior of these 2 enzymes suggests that they are responsible for ammonia assimilation, while the NADPH dependent glutamate dehydrogenase is not. The difference in response to nitrate and ammonia of the NADH and NADPH glutamate dehydrogenases, and their changing ratios, suggests that they are separate enzymes, E.C. 1.4.1.2 and E.C. 1.4.1.4. No attempt was made to assay separately the cyto-

plasmic and particulate dehydrogenases, although it is known, for example in pea roots (unpublished results) that NADH and NADPH activities are found in both cytoplasmic and particulate fractions, and that the properties of the enzymes are very different. In this work the addition of a detergent ensured that particulate as well as cytoplasmic glutamate dehydrogenases would be assayed.

Recent work by Sims and Folkes (9) shows that glutamate dehydrogenase in yeast is susceptible to both feedback inhibition and repression by a range of amino acids. They have shown that under some conditions of yeast culture the high rate of amino acid synthesis and accumulation with ammonia as nitrogen source is sufficient to repress glutamate dehydrogenase, so that the measured levels of the enzyme are actually lower on ammonia than on nitrate, where lower levels of amino acid in the cells apparently allows greater expression of the enzyme. Similar results were found here for *Lemna*, with higher activities of glutamate dehydrogenase on nitrate than on ammonia media (table I), and this may be explained in the same way. In contrast to this result, however, it was found that while transfer from amino acid media to ammonia caused considerable increase in activity of glutamate dehydrogenase (NADH), no such increase was found with nitrate. This is readily explained, assuming that glutamate dehydrogenase is indeed induced by ammonia, and repressed by amino acids. The amino acid grown tissue would undoubtedly contain high concentrations of soluble amino acids, which would repress glutamate dehydrogenase, and which might decrease only slowly after transfer to different media. On transfer to ammonia, a high concentration of ammonia would accumulate in the tissue, thus achieving (at least by the second day, Fig. 1) a satisfactory ratio of ammonia to amino acids and allowing enzyme induction to occur. With nitrate however there would be only slow accumulation of ammonia, since input would be controlled by nitrate reductase, and furthermore the existing levels of glutamate dehydrogenase, although low, would reduce some of the ammonia as it was formed. Thus a ratio of ammonia to amino acids suitable for induction would be attained very slowly when nitrate was the nitrogen (and thus ammonia) source following culture on amino acids.

It is attractive to assume that in plants amino acids may exert some control over the metabolic pathways leading to their synthesis. Some limited experiments investigating amino acid effects on enzyme induction have given results which are ambiguous in view of the toxic effects of amino acids noted earlier (5). Control of nitrate reductase by amino acids has been found in tobacco tissue (2). While no direct evidence for enzyme control by amino acids has been clearly shown yet in the work with *Lemna*, some of the results are consistent with this assumption; for example, the low levels of glutamate dehydrogenase found in amino acid grown tissue, and the subsequent behavior on transfer to an alternative nitrogen source, as discussed above. In addition, it was found that nitrite was much more toxic in the presence of amino acids, and this would be readily explained if nitrite reductase was repressed by amino acids so preventing reduction, and thus detoxification, of nitrite entering the cell.

Whether or not amino acids may play a role in regulation of the pathway, it is already apparent that for *Lemna*, a higher plant, a complex system exists for the regulation of the enzymes of nitrate and ammonia assimilation.

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